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Antibiotic Susceptibility of the Newly Cultivated Agent of Human Granulocytic Ehrlichiosis: Promising Activity of Quinolones and Rifamycins

MARINA B. KLEIN, CURTIS M. NELSON, AND JESSE L. GOODMAN*

Department of Medicine, Division of Infectious Diseases, University of Minnesota, Minneapolis, Minnesota

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Human granulocytic ehrlichiosis (HGE) is a rapidly emerging tick-borne infection which presents as an acute febrile illness and is associated with hematologic abnormalities, elevated hepatic transaminase levels, and characteristic intracellular organisms in peripheral blood granulocytes. Although HGE has been successfully treated with tetracyclines, its susceptibility to other antibiotics remains unknown. No clear treatment alternatives exist for young children, pregnant women, or allergic individuals, in whom tetracyclines are contraindicated. We performed in vitro antibiotic susceptibility tests with this recently isolated agent grown in the human promyelocytic leukemia cell line HL-60. Doxycycline (MIC, 0.25 μg/ml), rifampin (MIC, 0.5 μg/ml), rifabutin (MIC, ≤0.125 μg/ml), ciprofloxacin and ofloxacin (both with MICs of 2 μg/ml), and trovafloxacin (MIC, ≤0.125 μg/ml) demonstrated significant activity against the HGE agent. These agents were also bactericidal. The HGE agent was resistant to clindamycin, trimethoprim-sulfamethoxazole, and imipenem-cilastatin, as well as to ampicillin, ceftriaxone, erythromycin, and azithromycin, antibiotics commonly used to treat Lyme disease. Both chloramphenicol and gentamicin had weak inhibitory activities but were not bactericidal. Our findings confirm the observed clinical efficacy of doxycycline and further suggest that the rifamycins and quinolones, particularly trovafloxacin, hold promise as alternative agents for treating this new infection.

Human granulocytic ehrlichiosis (HGE) is a rapidly emerging infection first recognized in Minnesota and Wisconsin in 1994 (2, 7). In the past 2 years increasing numbers of cases (at last count, more than 200) have been reported from the Midwest and more recently from New York and Massachusetts (6, 21). HGE is caused by an obligate intracellular gram-negative bacterium which is closely related, if not identical to, the ehrlichiae infecting horses and cattle, *Ehrlichia equi* and *Ehrlichia phagocytophila* (7, 8, 10). HGE is most likely transmitted by *Ixodes* ticks, also vectors of Lyme disease and babesiosis (18). Coinfection with the HGE agent and *Borrelia burgdorferi*, the agent of Lyme disease, as well as with *Babesia microti*, has been reported (14, 16).

The clinical presentation of HGE is characterized by the acute onset of fever, myalgias, and headache. Associated laboratory features include thrombocytopenia, leukopenia, and elevated hepatic transaminase levels. Intracellular organisms may be observed in peripheral blood granulocytes, but may be absent in a substantial proportion of patients, making empiric antibiotic therapy necessary pending confirmatory serologic or PCR testing. Infection may be severe and deaths have been reported in up to 5% of patients (1). Of 41 patients with HGE recently reviewed, 33 were treated with doxycycline, 31 of whom recovered without sequelae (1). There is little reported clinical experience with other antimicrobial agents, and no clear treatment alternatives are available for pregnant women, young children, and allergic individuals, in whom tetracyclines are contraindicated.

Studies of the HGE agent have until recently been impossible due to the inability to cultivate the organism. Our laboratory recently described the in vitro cultivation of the etiologic agent of HGE in the human promyelocytic leukemia cell line HL-60 (10). This has allowed for the performance of in vitro antibiotic susceptibility tests with this organism and the identification of antimicrobial agents with potential clinical activity against this emerging disease.

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* Corresponding author. Mailing address: University of Minnesota, Division of Infectious Diseases, 516 Delaware St. SE, Box 250 UMHC, Minneapolis, MN 55455. Phone: (612) 624-9996. Fax: (612) 625-4410. E-mail: jesse@lenti.med.umn.edu.

MATERIALS AND METHODS

Isolation and cultivation of HGE. Three strains of HGE were used (one each from Minnesota, Wisconsin, and New York). These strains were isolated in our laboratory from the blood of patients with acute ehrlichiosis, identified as HGE by genospecies-specific PCR, and were propagated continuously in the human leukemia cell line HL-60 (CL240; American Type Culture Collection) as described previously (10). Cells were maintained at a density of between 2×10^5 and 1×10^6 cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine at 37°C with 5% CO_2.

Giemsa staining to determine degree of infection. Cytospin slides were prepared by using 100 μl of untreated control or antibiotic-treated cultures, air dried, fixed in methanol for 10 min, and Giemsa stained (pH 6.8) for 30 min. Slides were viewed under oil immersion (×1,000 magnification), and 100 cells were examined to determine the percentage containing intracellular bacteria (morulae) (e.g., see Fig. 1A, showing 25% infected cells).

Antibiotics. Stock solutions of doxycycline (Fujisawa, Deerfield, Ill.), ciprofloxacin (Miles Inc., West Haven, Conn.), ofloxacin (McNeill Pharmaceutical, Spring House, Pa.), chloramphenicol (Sigma Chemical Co., St. Louis, Mo.), clindamycin (Upjohn, Kalamazoo, Mich.), and gentamicin (Life Technologies Inc., Grand Island, N.Y.) were prepared from aqueous solutions immediately before use. Trovafloxacin mesylate (CP-99,219; Pfizer, Groton, Conn.), ampicillin (Sigma), ceftriaxone (Hoffmann-La Roche, Nutley, N.J.), and imipenem-cilastatin (Merck Sharp & Dohme, West Point, Pa.) were prepared from powdered bases by dissolving them in culture medium. Erythromycin and rifampin (Sigma), rifabutin (Pharmacia, Albuquerque, N.M.), and azithromycin (Pfizer) powdered bases were dissolved initially in 95% ethanol and were then brought up to the desired stock concentration by using culture medium. Trimethoprim (Sigma) was dissolved in water and was heated to 50°C, and 1 N HCl was slowly added until the compound completely solubilized. Sulfamethoxazole (Sigma) was mixed with water, and 1 M NaOH was added until it dissolved. Trimethoprim and sulfamethoxazole were then combined in a 1:19 ratio for testing. All stock

TABLE 1. Antibiotics with significant anti-HGE activity

Antibiotic	MIC (μg/ml)	MBC (μg/ml)	NCCLS MIC for susceptibility (μg/ml)[a]
Doxycycline	0.25	0.25–0.5	≤4
Ciprofloxacin	2	2–4	≤1
Ofloxacin	2	2–4	≤2
Trovafloxacin	≤0.125	0.125–0.5	NA[b]
Rifampin	0.5	0.5–1	≤1
Rifabutin	≤0.125	0.5–1	NA

[a] Data are from reference 22.
[b] NA, not available.

antibiotic solutions were filter sterilized by using 0.2-μm-pore-size low-protein-binding syringe filters (Acrodisc; Gelman Sciences, Ann Arbor, Mich.). The highest concentration of each antibiotic tested was chosen to be at least equal to the MIC breakpoint for resistance defined by the National Committee for Clinical and Laboratory Standards (NCCLS) (22). Decreasing serial dilutions were made to concentrations below the NCCLS MIC breakpoint for susceptibility. We tested the following antibiotics at the indicated concentrations: doxycycline, 0.06 to 16 μg/ml; rifampin, rifabutin, ciprofloxacin, trovafloxacin mesylate, and clindamycin, 0.125 to 4 μg/ml; ofloxacin, 0.125 to 8 μg/ml; erythromycin and azithromycin, 0.25 to 8 μg/ml; trimethoprim-sulfamethoxazole, 0.25/4.75 to 16/304 μg/ml; ampicillin, imipenem-cilastatin, and chloramphenicol, 4 to 32 μg/ml; ceftriaxone, 4 to 64 μg/ml; and gentamicin, 50 μg/ml.

Susceptibility testing. When HL-60 cells were approximately 25% infected, 0.5 ml of a culture containing 6×10^5 cells/ml was seeded into each well of a 24-well tissue culture plate, and then 0.5 ml of a 2× concentration of antibiotic solution was added to achieve a final volume of 1 ml containing the test concentration of antibiotic and 3×10^5 cells/well. Control wells containing no antibiotic were prepared in parallel. Doxycycline was used as a positive control in all experiments. The cells were incubated in 5% CO_2 at 37°C. Preliminary experiments in which samples were taken daily from the time of incubation with antibiotics revealed that maximal inhibition of infection occurred by day 3 (data not shown). Therefore, in all subsequent experiments, cells were incubated with antibiotics for 72 h, and then the wells were sampled and the slides were Giemsa stained. The MIC of each antibiotic was defined as the lowest antibiotic concentration resulting in greater than 90% reduction of infected cells. The cells were then washed free of antibiotic by removing the contents of each well, adding 3 ml of fresh medium, and pelleting by centrifugation at $200 \times g$ for 5 min. The cell pellets were washed a second time by resuspension in 4 ml of fresh medium and were again centrifuged as described above. The washed cells were replated in 1 ml of fresh antibiotic-free medium. The plates were then incubated without antibiotics for an additional 11 days, at which time samples were taken to determine a minimal bactericidal concentration (MBC), which was the lowest antibiotic concentration resulting in the complete absence of infection. Each antibiotic was tested at least twice against the same HGE isolate in separate experiments. Antibiotics demonstrating significant activity were tested against all three HGE isolates to determine the consistency of their activity against geographically diverse strains.

RESULTS

The agent of HGE was highly susceptible to doxycycline (MIC, 0.25 μg/ml), rifampin (MIC, 0.5 μg/ml), and rifabutin (MIC, ≤0.125 μg/ml). In addition, variable activity was noted among those quinolones tested, with trovafloxacin mesylate (MIC, ≤0.125 μg/ml) being the most active (Table 1). With the exception of ciprofloxacin, antibiotics demonstrating significant activity were both inhibitory and bactericidal below the limits of susceptibility outlined by NCCLS. By NCCLS criteria, the HGE agent would be considered sensitive to ofloxacin (MIC, 2 μg/ml) and intermediately sensitive to ciprofloxacin (MIC, 2 μg/ml).

The HGE agent was highly resistant to ampicillin, ceftriaxone, imipenem-cilastatin, erythromycin, azithromycin, clindamycin, trimethoprim-sulfamethoxazole, and gentamicin (Table 2). Chloramphenicol inhibited infection by 75% at the maximum concentration tested (32 μg/ml), but was not bactericidal. Similarly, 50 μg of gentamicin per ml demonstrated some inhibitory activity (50%), but was not bactericidal.

Due to the variability inherent in a dynamic culture system, the starting percentage of infected cells used in each experiment ranged from 11 to 42% (mean, 23%). MIC determinations were highly reproducible in all experiments and with all three isolates tested, regardless of the initial degree of infection in the cells used. MBC determinations varied within 2 dilutions, depending on the initial level of infection (i.e., >25% initial infection led to a higher final MBC and vice versa). MBC results are therefore reported as ranges.

Figure 1 shows HGE-infected cells prior to treatment (Fig. 1A) and 3 days after treatment with medium alone (Fig. 1B), doxycycline (Fig. 1C), trovafloxacin (Fig. 1D), rifabutin (Fig. 1E), and ampicillin (Fig. 1F). Treatment with medium and ineffective antibiotics such as ampicillin resulted in near complete infection and lysis of HL-60 cells by the bacteria within 3 days.

DISCUSSION

We report on the development and use of an in vitro culture system to determine the antibiotic susceptibility of the HGE agent. The HGE agent was found to be highly susceptible to doxycycline, confirming the observed clinical efficacy of this antibiotic. HGE was, however, resistant to antibiotics representative of those commonly used in the treatment of Lyme disease (e.g., ampicillin, ceftriaxone, erythromycin, and azithromycin). Given the known activity of doxycycline against *B. burgdorferi* and the possibility of coinfection with both bacteria, our findings strongly support the use of doxycycline, whenever possible, as the first-line antibiotic for the treatment of both infections, especially when using empiric treatment.

Because illness caused by HGE can be very serious, alternative therapies are urgently needed. Tetracyclines are contraindicated during pregnancy and are not widely accepted for use in children under 9 years of age because of the risk of staining permanent teeth and impairing bone growth. Serious allergic reactions to tetracyclines, while rare, do occur. Our results suggest that both rifamycins and quinolones, particularly the developmental fluoroquinolone trovafloxacin mesylate (CP-99,219), have significant anti-HGE activity. In vitro studies of *Ehrlichia sennetsu* (5), *Ehrlichia canis* (4), and *Ehrlichia chaffeensis* (3) have previously shown rifampin to be highly active, but we are unaware of any studies in which rifamycins were used in the treatment of human ehrlichiosis. Both rifampin and rifabutin were very active (MICs, 0.5 and ≤0.125 μg/ml, respectively) against HGE and can be safely used in children, making them attractive alternatives to doxycycline. Although used for the treatment of tuberculosis during pregnancy, their safety remains unproved in this setting. Ciprofloxacin has been reported to be very active (MIC, ≤0.125

TABLE 2. Antibiotics without significant in vitro activity against the HGE agent

Antibiotic	Maximum concn tested (μg/ml)	NCCLS MIC for susceptibility[a] (μg/ml)
Chloramphenicol	32[b]	≤8
Ampicillin	32	≤8
Ceftriaxone	64	≤8
Imipenem-cilastatin	32	≤4
Erythromycin	8	≤0.5
Azithromycin	8	≤2
Clindamycin	4	≤0.5
Gentamicin	50[b]	≤4
Trimethoprim-sulfamethoxazole	16/304	≤2/38

[a] Data are from reference 22.
[b] Some inhibitory activity was noted at these concentrations (see text).

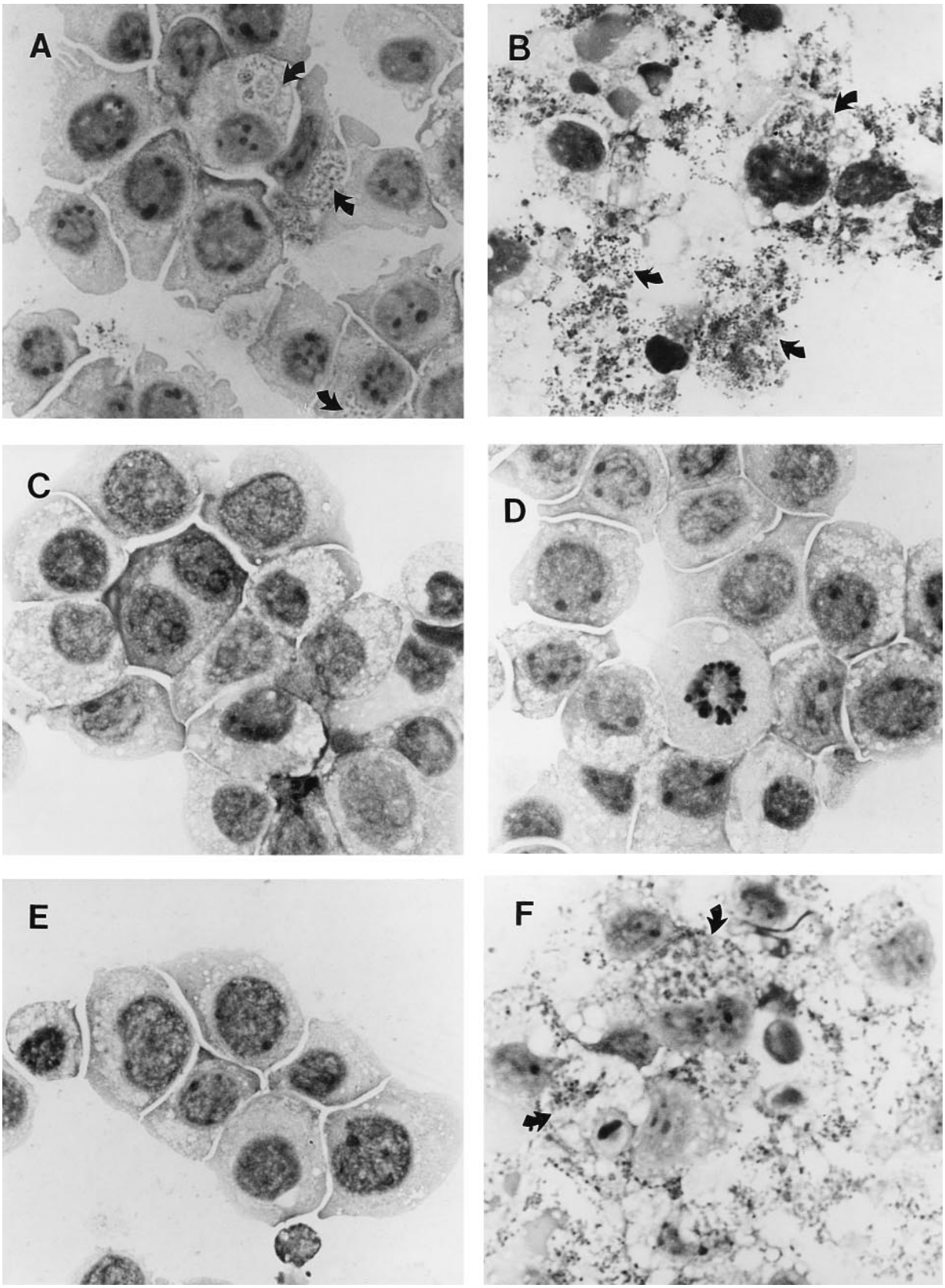

FIG. 1. Giemsa-stained HL-60 cells. (A) Starting culture infected 25% and cells 3 days after incubation with the following representative antibiotics and controls: infected untreated control (B), doxycycline at 0.25 μg/ml (C), trovafloxacin at 0.125 μg/ml (D), rifabutin at 0.125 μg/ml (E), and ampicillin at 32 μg/ml (F). Arrows indicate representative bacterial forms. Final magnification, ×3,168.

μg/ml) against *E. sennetsu* (5) and *E. canis* (4), but not against *E. chaffeensis*, the agent of monocytic ehrlichiosis in the United States (3). We found that the MIC of ciprofloxacin (2 μg/ml) was greater than the NCCLS susceptibility breakpoint (1 μg/ml), raising concerns about its usefulness in the treatment of HGE. The MIC of ofloxacin (2 μg/ml) was equal to its NCCLS breakpoint. Trovafloxacin was significantly more active (MIC, ≤0.125 μg/ml) and may hold considerable promise for the treatment of this infection. Trovafloxacin has been shown to have equal or greater activity compared with those of ciprofloxacin and newer agents, such as sparfloxacin, tosufloxacin, and temafloxacin, against a variety of organisms, including intracellular pathogens such as *Listeria monocytogenes* and *Yersina enterocolitica* (17). Concerns have been raised by reports of articular damage in juvenile animals treated with quinolones. For this reason, use of these antimicrobial agents has been considered contraindicated in prepubertal children and pregnant women. Recent reviews (15) and a consensus statement (20) have highlighted the absence of documented articular toxicity in more than 1,500 children treated with quinolones, and new recommendations allow for the cautious use of quinolones in this population when deemed clinically necessary.

We previously reported a successful outcome when we used chloramphenicol to treat a severely ill patient with confirmed HGE and a prior history of anaphylaxis following the administration of tetracycline (10). In previous in vitro studies, chloramphenicol was found to have poor activity against *E. sennetsu* (5), *E. canis* (4), and *E. chaffeensis* (3), consistent with our finding of an MIC of ≥32 μg/ml for HGE. Since chloramphenicol is concentrated in neutrophils with cellular concentration to extracellular concentration ratios of 2 to 3 (13, 19) and reaches phagocytic vacuoles in its active form (13), it is possible that in vivo clinical efficacy could occur despite poor in vitro MICs. In fact, despite the poor in vitro activity of chloramphenicol against *E. chaffeensis*, all seven patients who received the drug in one retrospective case series of monocytic ehrlichiosis fared as well as those receiving tetracyclines with regard to duration of fever, need for hospitalization, and time to recovery, and fared statistically better than those receiving antibiotics other than tetracycline or chloramphenicol (9). In addition, three children with monocytic ehrlichiosis were reported to have improved rapidly following treatment with chloramphenicol (11).

Gentamicin had some inhibitory activity against HGE at 50 μg/ml, a level not obtainable in human blood without severe toxicity. Given this finding, it would be prudent not to use aminoglycosides as antibiotics in tissue culture when trying to recover HGE. We routinely perform cultures of blood for HGE in the absence of any antibiotics.

In conclusion, doxycycline, quinolones, and rifamycins have significant in vitro bactericidal activity against the causative agent of HGE. The susceptibility patterns did not vary among isolates from different geographic regions. Studies with animal models, when they become available (12), and patients are needed to further validate these in vitro findings and to define the potential roles of quinolones and rifamycins in the treatment of infections due to this important new agent.

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